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MECHANISM OF DEATH OF T47D BREAST CANCER CELLS AGAINST DICHLOROMETHANE FRACTIONS FROM KANDIS CORTEX (*GARCINIA COWA* ROXB)

Roslinda Rasyid, Fatma Sriwahyuni and Harrizul Rivai*

Faculty of Pharmacy, Andalas University, Limau Manih Campus, Padang 25163, Indonesia.

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***Corresponding Author**
Harrizul Rivai
Faculty of Pharmacy,
Andalas University, Limau
Manih Campus, Padang
25163, Indonesia.

ABSTRACT

Breast cancer is a leading disease that causes death among woman in the world. Herbal medicine is often used as alternative medicine in cancer therapy. *Garcinia cowa* Roxb has been reported that has pharmacological activity, one of them is anticancer. Dichloromethane fraction of kandis stem bark has cytotoxic activity on T47D breast cancer cell with IC_{50} 4.07 µg/mL. The most potent cytotoxic activity of fraction is fraction dichloromethane. Apoptosis and necrosis examination was conducted by double staining method using acridine orange-propidium iodide and observed by using a fluorescence microscope after 24 hours of incubation. Dichloromethane fraction of the kandis stem bark can trigger breast cancer T47D cell line through apoptotic cell death mechanism. Dichloromethane fraction caused 29.92% apoptosis effects and 7.09% necrosis effect. *Garcinia cowa* Roxb could induce apoptosis on T47D breast cancer cell.

KEYWORDS: T47D Breast Cancer Cell, Double Staining, Apoptosis, Garcinia Cowa Roxb.

INTRODUCTION

A status report on the global burden of cancer worldwide estimates of cancer incidence and mortality produced by the International Agency for Research on Cancer, with a focus on geographic variability across 20 world regions. There will be an estimated 18.1 million new cancer cases (17.0 million excluding non-melanoma skin cancer) and 9.6 million cancer deaths (9.5 million excluding non-melanoma skin cancer) in 2018. In both sexes combined, lung cancer is the most commonly diagnosed cancer (11.6% of the total cases) and the leading cause of cancer death (18.4% of the total cancer deaths), closely followed by female

breast cancer (11.6%), prostate cancer (7.1%), and colorectal cancer (6.1%) for incidence and colorectal cancer (9.2%), stomach cancer (8.2%), and liver cancer (8.2%) for mortality. Lung cancer is the most frequent cancer and the leading cause of cancer death among males, followed by prostate and colorectal cancer (for incidence) and liver and stomach cancer (for mortality). Among females, breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death, followed by colorectal and lung cancer (for incidence), and vice versa (for mortality); cervical cancer ranks fourth for both incidence and mortality.[1]

From previous studies, it has been known that kandis (*Garcinia cowa* Roxb) (Figure 1[2]) contains xanthone in the form of prenylated xanthone and oxygenated xanthone in almost all parts of the plant. It has also been found that xanthones and prenylated xanthones show the strong cytotoxic potential that can be used as a potential new cytotoxic agent.[3] From a study, it was also known that kandis fruit has an antioxidant and antimutagenic effect.[4]

Figure. 1: Tree of *Garcinia cowa* Roxb.[2]

Treatments for cancer, such as surgery, radiation, and chemotherapy, require very high costs. At present, in addition to treatment with chemotherapy, people also try many possibilities for healing with alternative medicine by using natural ingredients.[5]

In this study, we will examine the apoptosis or programmed cell death process. The double staining method saw changes in cell morphology after being given specific reagents. The reagent used is binding dye reagent, a reagent that can dye cells based on cell membrane integrity. The reagents used were orange acridine and propidium iodide. This research was carried out to continue the previous research on cytotoxics, in which the study examined

various extracts and fractions of the kandis stem skin in terms of killing cancer cells. The study found that the dichloromethane fraction, which gave the best IC50 value was 4.07 µg/mL.[6] This study used dichloromethane fraction from the skin of the kandis stem to determine T47D breast cancer cell death.

MATERIALS AND METHODS

Tools and materials: The tools used are rubber gloves, spray bottles, Erlenmeyer, microtube, T-25 flask (Iwaki®), Duran bottles, Eppendorf (Iwaki®) pipettes, micropipettes (Ecopipette®), hemasitometers, analytic scales, autoclaves (Hirayama®), 37 ^{o}C/5% CO_2 incubator (Thermo Scientific®), microbiological safety cabinet flow class II water (Thermo Scientific®), vortex (Etech®), water bath (Memert®), centrifuge (Thermo Scientific®), tube centrifuges, inverted microscopes (Zeiss®), Axio Imager A2 (Zeiss®) microscopes, slipcovers, pits 24 plates. The material used for cell death detection was dichloromethane fraction of kandis stem skin (*Garcinia cowa* Roxb), T47D human breast cancer cells collected by Cancer Chemoprevention Research Center, dimethyl sulfoxide (DMSO), 70% ethanol, ultrapure water, culture media from Roswell Park Complete Memorial Institute (RPMI) 1640, Trypsin-EDTA, Phosphate Buffer Saline (PBS), Acridine orange (AO), Propidium Iodide (PI).

Procedure

Tools preparation: The tools used for testing must be clean and sterile. Plastic containers are prepared for only one use, and the sterility is guaranteed as long as the packaging is not damaged. For glassware, the container is washed and dried. Then the tools were sterilized by autoclaving at 121 ^{o}C at a pressure of 15 lbs for 15 minutes. While the microbiological safety cabinet airflow class II is sterilized by spraying with 70% ethanol.

Cell preparation: The breast cancer cells used are T47D cells, a collection of the Gajah Mada University Cancer Chemoprevention Research Center (CCRC). Cancer cells are removed from the freezer (-80 oC), warmed in a water bath at 37 ^{o}C for 2-3 minutes. After melting, the cells are transferred into a 5 ml flask, incubated for 3-4 hours at 37 °C, 5% CO_2, then observed under a microscope to see if the cells attach to the base of the flask and form a monolayer layer. The growth medium is replaced once every two days, and if the number of cells in the flask reaches 70-85%, subcell culture is carried out.

Cell subculture: The medium in the flask was removed, then added 2 ml of trypsin-EDTA then stirred slowly, incubated for 5-15 minutes at 37 ^{o}C, 5% CO_2. After that, the cell was observed under a microscope. Cells that are ready to use will float and separate from the colony. Then the trypsin-EDTA solution containing the cell was centrifuged at 3000 rpm for 5 minutes. The supernatant was removed, then the remainder was suspended in 3 mL medium and put into a new flask, stirred slowly and incubated at 37 °C, 5% CO_2.

Cell count: Two mL of trypsin-EDTA was added into a flask containing cell culture, then incubated for 5-10 minutes. Then the trypsin-EDTA solution containing the cell was centrifuged at 3000 rpm for 5 minutes, the supernatant was removed, then the remainder was suspended in 3 ml of RPMI medium. A total of 10 µL of cell suspension was placed in each of the squamous cell counting boxes. Cell counts are carried out under a microscope, and the average number of cells is determined to make cell suspension. Fifty thousand cells in each well of the 24 well flat bottom cell culture plates were carried out for analysis of cell death detection.

Cell laying: Cell suspension in the medium is diluted to reach a concentration of 5 x 10^4 cells/mL in each well of the culture medium. Prepare the well plate and coverslip into the well using tweezers carefully. Transfer 1000 µL of cell suspension onto the coverslip that has been inserted into the well by inserting 200 µL of cell suspension onto the coverslip, then incubating 30 minutes, then adding 800 µL of cell suspension This is done so that the cell attaches to the coverslip. The first column is a control containing cell suspension and media, and the next column is cell suspension to be treated with dichloromethane extract at a concentration of 4 µg/mL. Incubation at 37 ^{o}C, 5% CO_2 for 24 hours. If within 24 hours, the cell condition has not been attached, replace the cell media and re-incubated.

Preparation of test solutions

a. Stock solution: As much as 100 mg of dichloromethane fraction from the extract of the kandis bark were weighed and dissolved in 1 mL of the appropriate solvent to obtain a solution concentration of 100 mg/mL. The dichloromethane fraction must be completely soluble in the solvent used.

b. Test solution for detection of cell death: One Eppendorf tube is prepared for each sample, labeled according to the concentration to be made. Dilution was carried out to obtain a concentration of 4 µg/mL in the RPMI-1640 media.

Test the cause of cell death

a. Laying the test solution: After the cells are attached to the bottom of the well and are confluent, a dilution of one sample concentration is immediately made at four µg/mL IC_{50} for the treated column. Discard all the media in the well with the Eppendorf pipette slowly, fill PBS with 500 µl each, then dispose of PBS with the Eppendorf pipette, and then insert the sample into the well. In the control column, only 1000 µL of media is given. Incubate in an incubator for 24 hours.

b. Double staining method using a fluorescence microscope

After incubation is complete, remove the plate from the incubator. Gently remove all media with the Eppendorf pipette. Fill PBS into each well as much as 500 µL, then slowly remove PBS with the Eppendorf pipette. Take the coverslip with tweezers with the help of the tip of the needle carefully. Place it on a glass object. The position of the cell must be above, not to the upside. After that, on the coverslip, a working solution of acridine orange (AO) and propidium iodide (PI) was added to each 10 µL, smoothing it by gently rocking it. Cells were observed under the microscope Axio Imager A2 with fluorescence settings (Zeiss®). Two hundred cells were analyzed under a fluorescence microscope. Repetition is done three times. Morphology of cell viability, necrosis, and cell apoptosis will be photographed and analyzed.

DATA ANALYSIS

The observations under the fluorescent microscope will show that living cells fluoresce green, cells undergoing apoptosis fluoresce yellow while cells are undergoing necrosis fluoresce reddish orange. Each cell that has apoptosis or necrosis is calculated in number. The number of cells from the results of these calculations, it can be determined the percentage of cells undergoing apoptosis or necrosis.

% apoptotic cells = (average apoptotic cells) / (total cell count) x 100%

% cell necrosis = (average cell necrosis) / (total cell number) x 100%

% cell viable = (average cell viable) / (total cell number) x 100%

RESULTS

The percentage of viable cells, apoptosis, and necrosis in cells not induced by dichloromethane fraction was 95.38 ± 4.77%, 3.43 ± 3.28%, 1.17 ± 1.59%, respectively (Figure 2 and 4).

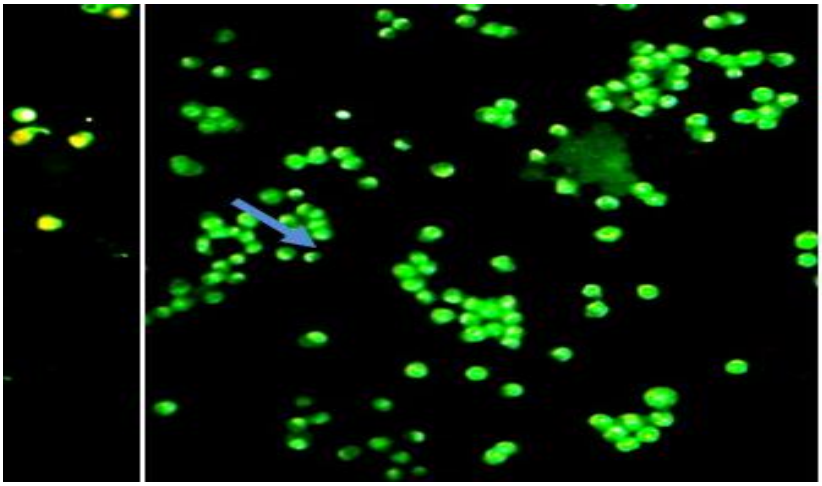

Figure. 2: Fluorescence of the color of the control cell (not given treatment with the kandis stem dichloromethane fraction) at incubation after 24 hours. Viable cells uniform green fluorescence (100x magnification).

The percentage of viable cells, apoptosis, and necrosis in cells induced by dichloromethane fraction at IC50 4 µg / mL was 62.97% ± 4.17, 29.92 ± 3.23, 7.09% ± 2.00, respectively (Figure 3 and 4).

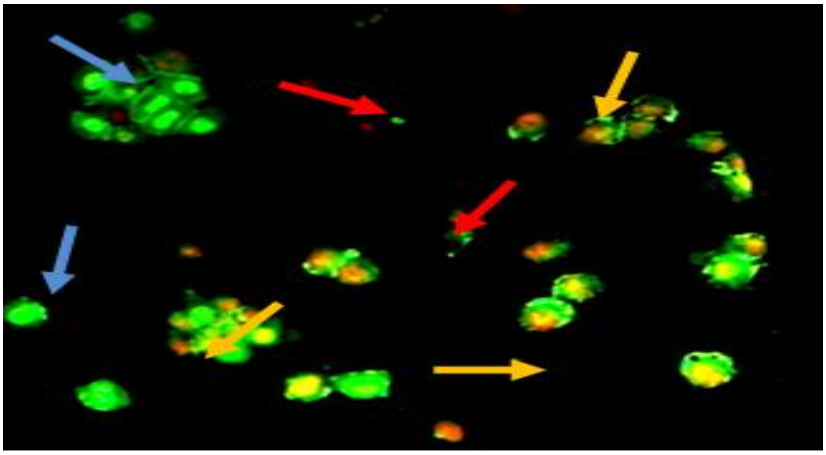

Figure. 3: Color fluorescence from the effect of dichloromethane fraction of kandis stems skin with IC50 4 µg/mL on viable cells, apoptosis and necrosis in T47D breast cancer cells at 24-hour incubation. Viable cells with uniform green fluorescence; cells that experience death by apoptosis yellowish green fluorescence; cells that experience death by necrosis floured orange to reddish) (100x magnification).

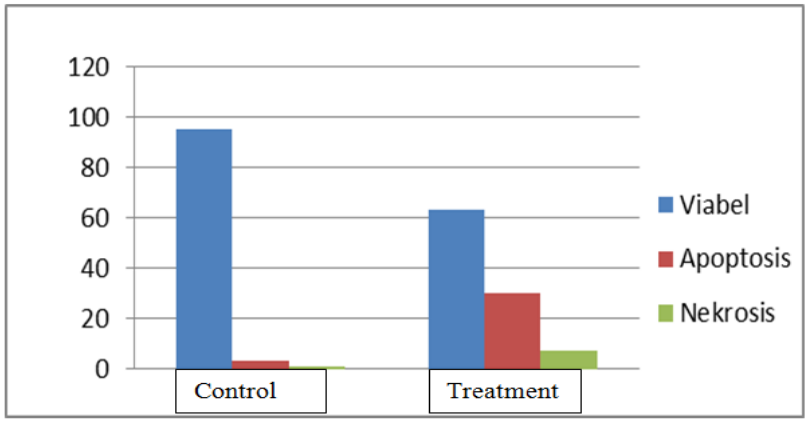

Figure. 4: Percentage of viable cells, apoptosis, and necrosis in the control and treatment with dichloromethane fraction of kandis bark extract.

DISCUSSION

Research on the detection of T47D breast cancer cell death is a continuation of previous studies, namely the cytotoxic test of the kandis stem skin fraction. From this study, the most active fraction was dichloromethane fraction with an IC50 value of 4.07 µg/mL. IC50 value shows the concentration value, which results in 50% cell proliferation resistance and shows the toxicity of a compound to cells.[7]

The test carried out in this study was a double staining test. DNA dyes used are acridine orange and propidium iodide. This method is based on differences in DNA fluorescence in cells that live and die due to binding of acridine orange - propidium iodide. Propidium iodide is a selective DNA color that cannot penetrate the membrane but can easily penetrate the plasma membrane that has undergone necrosis, often used for cell death testing. Akridin orange is permeable so that it can enter healthy cells and dead cells Acridine orange and propidium iodide will emit green fluorescence and red fluorescence when binding to DNA. Propidium iodide will fluoresce reddish orange if the cell has undergone necrosis. Propidium iodide - acridine orange can dye the nucleus in the apoptotic stage to be yellow.[8]

Calculations for viable cells, apoptosis, and necrosis are presented as a percentage. The total cell count for each coverslip is at least 200 cells. The calculation for apoptotic cells is the number of cells undergoing apoptosis divided by the total number of cells on the coverslip. Data were processed using SPSS 17.0 using two independent sample t-tests. The value of viable cell probability, apoptosis and necrosis have $P < 0.05$, which means that control cells and treated cells have very significant differences.

Changes in cell morphology by looking at color changes in cells are the most common techniques. One excellent method is used for identification and calculation of apoptosis[9] Other stains that can also determine viable cells and non-viable cells are trypan blue, but trypan blue cannot determine how cell death occurs because changes in cell morphology cannot be observed with this coloring.[10]

T47D cells are breast cancer cells that lose p53 gene function. The loss of p53 gene function in cells due to mutations at 194 residues, so that leucine changes to phenylalanine in the p53 protein which causes p53 not to function[11] (Schafer et al., 2000). It results in p53 in T47D cells losing function in cell cycle inhibition at the G1 and G2 phases as well as spurring the occurrence of apoptosis.

The occurrence of apoptosis in T47D breast cancer cells, possibly through the mitochondrial pathway, because T47D has lost its p53 gene function. Induction of apoptosis through the intrinsic pathway is associated with the release of cytochrome c. When cells lose their self-defense ability or experience stress, Bcl-2 and Bcl-x will disappear from the mitochondrial membrane and be replaced by pro-apoptotic protein groups such as Bax, Bak, and Bim. When Bcl-2 / Bcl-x decreases, there is an increase in the permeability of the mitochondrial membrane causing the release of several proteins that will activate the caspase cascade. One of these proteins is cytochrome c. Cytochrome c is a part of the respiratory chain that resides and dissolves between the inner membrane and the outer membrane of the mitochondria.[12] In the cytosol, cytochrome c binds to Apaf-1 (apoptotic activating factor-1) and activates caspase-9.

Based on the data from this study, it can be seen that the dichloromethane fraction of the kandis stem skin induces the occurrence of apoptosis in T47D breast cancer cells. The occurrence of induction of apoptosis is likely via the mitochondrial pathway, by inducing caspase 3 and caspase 9.

CONCLUSIONS

The death of T47D breast cancer cells from the dichloromethane fraction of the kandis stem skin (*Garcinia cowa* Roxb) includes apoptosis and necrosis. The percentage of viable cells, apoptosis, and necrosis in cells treated with dichloromethane fraction of kandis stem skin at IC50 4 µg / mL was 62.97 ± 4.17%, 29.92 ± 3.23%, 7.09 ± 2.00%, respectively. With the parametric test independent t-test $p < 0.05$, there was a significant difference between treated

cells and untreated cells. Dichloromethane fraction from kandis bark extract with IC50 4 µg/mL can induce apoptosis in T47D breast cancer cells.

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REFERENCES

1. Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians, 2018; 68(6): 394-424.
2. https://www.jircas.affrc.go.jp/project/value_addition/Vegetables/054.html.
3. Jabit, M. L., Wahyuni, F. S., Khalid, R., Israf, D. A., Shaari, K., Lajis, N. H., & Stanslas, J. Cytotoxic and nitric oxide inhibitory activities of methanol extracts of Garcinia species. Pharmaceutical biology, 2009; 47(11): 1019-1026.
4. Wahyuni, F.S., Jubahar, J., Dianita, R., Dachriyanus, Lajis, N.H., and Sargent, M.V. (2003). Biological Activity and Chemical Constituents Studies of Garcinia cowa. National Symposium on Natural Product XIII. Structural Bioorganic Chemistry for Indonesia. Padang, 2003.
5. Djajanegara, I., & Wahyudi, P. Uji Sitotoksisitas Ekstrak Etanol Herba Ceplukan (Physalis angulata Linn.) terhadap Sel T47D Secara In Vitro. Jurnal Ilmu Kefarmasian Indonesia, 2010; 8(1): 41-47.
6. Rasyid, R, Susanti, M. & Gusmiwalti, I. Development of Analytical Method of α-mangosteen in Dichloromethane Extract of Green Fruit Latex Garcinia mangostana L. Using High-Performance Liquid Chromatography. Der Pharma Chemica, 2016; 8(16): 52-57.
7. Meiyanto, E., Agustina, D., Suparjan, A. M., & Da, I. M. PVG-O induces apoptosis on T47D breast cancer cells line through caspase-3 activation. Jurnal Kedokteran Yarsi, 2007; 15(2): 075-079.
8. Steigerová, J., Oklešťková, J., Levková, M., Rárová, L., Kolář, Z., & Strnad, M. Brassinosteroids cause cell cycle arrest and apoptosis of human breast cancer cells. Chemico-biological interactions, 2010; 188(3): 487-496.

9. Doonan, F., & Cotter, T. G. Morphological assessment of apoptosis. Methods, 2008; 44(3): 200-204.
10. Allen, R. T., Hunter III, W. J., & Agrawal, D. K. Morphological and biochemical characterization and analysis of apoptosis. Journal of pharmacological and toxicological methods, 1997; 37(4): 215-228.
11. Schafer, J. M., Lee, E. S., O'Regan, R. M., Yao, K., & Jordan, V. C. The rapid development of tamoxifen-stimulated mutant p53 breast tumors (T47D) in athymic mice. Clinical Cancer Research, 2000; 6(11): 4373-4380.
12. Green, D. R., & Reed, J. C. Mitochondria and apoptosis. Science, 1998; 281(5381): 1309-1312.